

Toxicity & Teratogenicity studies in Avian Embryos
Amaranth

No Date

FDA Contract #71-330

11

AMARANTH

Toxicity and Teratogenicity Studies
in Avian Embryos

FDA Contract #71-330

B.L. Reid

Department of Poultry Science
University of Arizona, Tucson, Arizona 85721

STUDIES ON THE
TOXICITY AND TERATOGENICITY OF
AMARANTH IN AVIAN EMBRYOS

Summary and Conclusions

Amaranth administrations into avian egg yolks at either zero or 96 hours incubation resulted in significant increases in embryo mortality ($P < 0.005$). A dose level of 0.2 mg./kg by yolk administration at 0 hours had no effect on embryo mortality while the next highest dose tested (2.0 mg./kg.) produced a significant mortality response ($P < 0.01$). Yolk administration at 96 hours of 40 mg. amaranth/kg. produced a significant ($P < 0.005$) increase in mortality. The no-effect dose for the yolk injection route for zero hour administration was between 0.2 and 2.0 mg./kg. while for 96 hour administration it was between 40.0 and 20.0 mg./kg.

Teratogenic effects of amaranth were linearly related to dose level for the yolk-96 hour series. A statistically significant increase in abnormal embryos was observed with the 20.0 mg./kg. dose level administered in the air cell at zero hours due to the histopathological findings.

Statistical analyses of teratology incidence in amaranth groups in comparison with all controls (solvent, drilled or pierced and untreated) showed significant chi-square values ($P < 0.005 - 0.05$) for both air cell administration times and for yolk-96 hour injected eggs. Although these studies provide no unequivocal evidence that amaranth is teratogenic for the

avian embryo, the incidence of abnormalities in the amaranth treated eggs in comparison with the controls requires further investigation.

GENERAL PROCEDURES

The protocols as specified under FDA Contract #71-330 were followed in the investigation of toxicity and potential teratogenicity of the specified substance. The toxicity of the substance was evaluated from the percentage hatch of embryos injected either in the air cell or yolk at either zero hours (post-incubation) or after 96 hours incubation to provide four separate evaluations.

EGG SOURCE AND HANDLING

All eggs used in these investigations were from Shaver Starcross pullets housed at the Poultry Research Center of the University of Arizona in Tucson. The parent stock was maintained on the University of Arizona breeder diet which had been formulated to provide more than adequate amounts of all the known nutrients required by the breeding hen.

The feed was specially prepared to assure no contaminations and did not contain any additive drugs such as antibiotics. All eggs prior to use (within 48 hours of lay) were candled to remove any containing blood spots, abnormal air cells or abnormal shells, and only clean eggs ranging in weight from 23 - 26 ounces per dozen were used.

The supply flock was tested to assure the absence of Pullorum and Mycoplasma gallisepticum.

The eggs were incubated in forced draft Jamesway 252 machines with automatic temperature and humidity controls and an automatic turning device.

COMPOUND HANDLING FOR INJECTION

The substance tested was solubilized in a number of the prescribed solvents in order to determine the maximum concentrations which could be employed. Where possible, water was the solvent of choice. Maximum

injection volume was 0.05 ml. and all solvents and glassware were autoclaved prior to preparation of the solutions for use. The dose levels were administered with a microliter syringe using sterilized needles.

The preliminary range-finding studies using each of the administration routes and times were carried out with 10 - 25 eggs per dose level and included solvent controls, untreated controls and either drilled or pierced controls.

The actual dose-response protocol was carried out in two or more injections on different days to produce a minimum of 100 eggs at each dose level in five or more levels selected from the range-finding studies.

EXAMINATIONS OF EMBRYOS AND CHICKS

Eggs were candled daily and the dead embryos removed, examined and any abnormalities recorded. Five chicks from each dose level in each hatch were X-rayed to determine any skeletal abnormalities. Additional eggs injected at the approximate LD-50 level and an additional level below that were incubated and embryos at 8, 14, 17 days and hatch chicks removed for histopathological examinations.

In additional studies representative chicks from the dose-response protocol were saved. These chicks were housed in electrically-heated battery brooders with raised wire floors and fed University of Arizona diets. Feed consumption and growth rates were evaluated at 6 weeks of age and a sample of the birds sacrificed for gross and histopathological examinations.

The remaining birds in each group were maintained to 6 months of age and then sacrificed.

DATA HANDLING

All data were coded on forms provided by FDA for computer input. In addition to summaries of mortalities and abnormalities, a number of statistical evaluations were carried out. These statistical analyses included the following for both mortality and the incidence of abnormal embryos:

1. Chi-square tests for all dose levels and for each level against the solvent control.
2. Linear regression analyses + chi square test of linearity.
 - a. % response against dose
 - b. % response against log dose
 - c. log % response against dose
 - d. arcsin transformation against dose
 - e. arcsin transformation against log dose
3. Log dose against Probit using Finney's maximum likelihood method.
 - a. Where significant, the LD-30, 50, 70 and 90's were estimated with 95% confidence intervals.
4. One-way analyses of variance.
5. Linear regression with replication.

Amaranth was finally solubilized in distilled water for use in the various test protocols. A concentration of 40 mg. per milliliter was found to be the maximum attainable at room temperature and this was employed at an injection volume of 0.05 ml. to produce the maximum dose levels of 2.0 mg. per egg or 40.0 mg./kg.

RESULTS AND DISCUSSION

Mortality

The administration of amaranth in the air cell in amounts from 0.004 to 40.0 mg./kg. of egg prior to incubation produced embryo mortalities which were significantly different from the solvent controls only with dose levels of 0.02 and 20.0 mg./kg. (Table 1). However, when the combined dose level mortalities were treated against the solvent controls the chi-square test yielded a value of 9.821 (P 0.25) which was not statistically significant.

The embryo mortalities were obtained when amaranth was injected into the air cell after 96 hours incubation ranged from 2.85 to 9.42% (Table 2). Statistical analyses of these data failed to show a significant effect of the amaranth administrations on embryo mortality.

Rather high embryo mortalities were produced with yolk administration prior to incubation (Table 3). The dose levels of 2.0 mg./kg. and above yielded significant chi-square values with P 0.05 - 0.005 and a comparison of all levels with the solvent controls yielded a chi-square value of 20.884 with 5 D.F. (P 0.005).

Amaranth appeared to be less toxic when administered in the yolk after 96 hours incubation with only the highest dose level of 40.0 mg./kg. yielding a significant chi-square value in the 2 x 2 adjusted test (P 0.005) although the comparison of all levels with the solvent controls had a chi-square of 21.589 with 5 D.F. (P 0.005).

These data suggest that amaranth is embryo toxic with the no-effect dose varying with the administration route and time of incubation. Avian embryos during the earlier stages of incubations were more susceptible than those after 96 hours development. The apparent

no-effect dose was less than 20.0 mg./kg. for air cell administration post incubation; while no significant differences from solvent control mortality were obtained at the highest dose tested (40.0 mg./kg.) in the air cell at 96 hours.

The yolk administration - zero hour apparent no-effect dose for mortality was below 2.0 mg./kg.; while 96 hour injection by the same route yielded a no-effect dose below 40.0 mg./kg.

TERATOLOGY

Among the eggs injected with amaranth in the air cell at zero hours those receiving 20 mg./kg. showed a statistically significant ($P < 0.01$) increase in teratology due to the histopathological findings in this group. The histopathological examinations showed malacia in the brain, granulated cytoplasm in liver cells and renal artery hypertrophy in this group.

Chi-square analysis employing a 2 x 2 table did not indicate a significant increase in abnormal embryos as a result of amaranth administration in the air cell at 96 hours or yolk administration at either 0 or 96 hours. A comparison of all dose levels of amaranth for each time and each administration route failed to indicate a statistically significant increase in comparison with the solvent controls. Similar analyses of the combined dose level data compared with all controls for each route and time (solvent, untreated and drilled or pierced) yielded significant chi-square values of 10.95 ($P < 0.05$), 12.75 ($P < 0.005$) and 4.005 ($P < 0.05$) for the two air cell administration times and yolk-96 hours, respectively, the yolk-0 hours series was not significant. This type analysis may be open to some question since the untreated controls were included; therefore, the same type analyses were undertaken without these groups being included. The chi-square values resulting from these comparisons of all dose levels with the solvent and drilled or pierced groups were not statistically significant ($P > 0.05$).

The incidence of abnormal embryos was linearly related to dose level only in the series of yolk injected eggs at 96 hours (Table 7); this was true for all of the data transformations employed in the computer program by FDA. Chi-square analyses of H-L-S-V abnormalities associated with amaranth were not significantly ($P > 0.05$) different from the solvent controls (Table 8).

There is no unequivocal evidence as a result of these studies that amaranth is teratogenic for the avian embryo. The data do suggest that the incidence of abnormalities in comparison with all control groups (including the untreated eggs) is significantly increased with amaranth administration, while the solvent controls and drilled or pierced groups of eggs did not exhibit the same increase in abnormalities in comparison with the untreated eggs. The only abnormality associated with the untreated groups was one chick showing atoxia, while the drilled controls at zero hours showed two embryos with anophthalmia and the remaining drilled or pierced eggs did not produce any embryos showing teratology. Among the solvent (water) injected controls three embryos or chicks were found to be abnormal with two showing celosomia and one with a head abnormality (Table 9).

POST-HATCH DATA

Among chicks raised to 6 weeks and 6 months there were no discernable differences in hatch weight, average of either males or females at 6 weeks or 6 months of age or in feed consumption (Table 10). The average age at sexual maturity for the female chicks varied from 140 to 166 days, but this difference cannot be considered significant in the light of the number of pullets involved. Mortalities were not significantly different through the post-hatch data collection periods and the gross, X-ray and histopathological examinations failed to indicate an effect of the amaranth administration on any of these criteria.

TABLE 1

AMARANTH in WATER

SUMMARY

AIR CELL - 0 HOURS

Dose, ppm	No. Fertile	Mortality %	Total %	Abnormal		Abnormalities by category							Functional %	Functional #		
				Total %	H-S-V-L %	Head %	Skeletal %	Viscera %	Limbs %	Struc- tural %	Response %					
												#			#	#
0.0	33	30.30	10	3.03	1	3.03	1				3.03	1				
20.0	177	31.63	56	5.64	10	1.69	3	0.56	1		1.12	2		0.56	1	1.12 2
10.0	32	28.12	9	0		0										
2.0	89	25.84	23	2.24	2	1.12	1				1.12	1				
0.2	114	28.94	33	4.38	5	2.63	3	0.87	1		0.87	1	0.87	1		
0.02	83	36.14	30	1.20	1	0									1.20	1
0.04	55	27.27	15	1.81	1	0									1.81	1
0.00	133	18.79	25	0.75	1	0.75	1				0.75	1				
Unexposed	116	24.13	28	1.72	2	1.72	2								1.72	2
Unexposed	426	11.03	47	0.23	1	0									0.23	1

SUMMARY - ALL DOSE LEVELS

583	30.19	176	3.26	1.37	8	0.27	2	0.69	4	0.34	2	0.17	1	0.51	3
-----	-------	-----	------	------	---	------	---	------	---	------	---	------	---	------	---

TABLE 2

AMARANTH in WATER

SUMMARY

AIR CELL - 96 HOURS

Dose, ppm	No. Fertile	Mortality %	Total %	Abnormal		Head %	Skeletal %	Viscera %	Limbs %	Struc- tural %	Response %	Functiona %
				#	#							
40.0	138	9.42	1.44	2	0.72	1	0.72	1		0.72	1	
20.0	105	4.76	0.95	1	0.95	1	0.95	1				
10.0	105	7.61	0.95	1	0							0.95
2.0	100	7.00	1.00	1	1.00	1			1.00	1		
0.2	105	2.85	0.95	1	0							0.95
0.0	129	4.65	0.77	1	0.77	1		0.77	1			
illed	151	2.64	0		0							
reated	426	11.03	0.23	1	0							0.23

SUMMARY - ALL DOSE LEVELS

553	10.13	56	1.08	6	0.72	3	0.36	2		0.18	1	0.18	1	0.18	1	0.36
-----	-------	----	------	---	------	---	------	---	--	------	---	------	---	------	---	------

Table 10

AMARANTH

POST HATCH DATA

Label	Dose, mg./kg.	Average age at Sexual Maturity	Average Body Weights, gm.				Average Feed Consumption/bird	
			At Hatch	6 weeks		6 months	6 wks., gm.	6 months kg.
				M	F	M		
1	20.0	156	42.6	398	397	1852	1702	858
2	2.0	147	42.3	465	382	2043	1816	849
3	0.2	166	43.0	439	418	1816	1703	841
4	0.02	140	43.4	459	407	2329	1816	845
5	0.004	145	42.5	464	413	2610	1893	912
8	Untreated	144	43.3	489	436	1852	1703	962

Table 9 cont'd

AMARANTH

SPECIFIC FINDINGS

Table 9 cont'd
(Sheet 2 of 3 sheets)

AMARANTH
TERATOGENIC FINDINGS

TREATMENT	TOTAL NO. EXAMINED	TOTAL NO. ABNORMAL	SPECIFIC FINDINGS												
			NO.	D	E	S	C	R	I	P	T	I	O	N	
Air Cell - 96 hours															
40.0	138	2	1												Anophthalmia, unilateral, dysallilognathia, dipygus Brain-malacia
20.0	105	1	1												Dysgnathia
10.0	105	1	1												Embryo malpositioned
2.0	100	1	1												Leg joint edema
0.2	105	1	1												Embryo malpositioned
0.00	129	1	1												Celosomia
Yolk - 0 hours															
40.00	107	4	1												Celosomia
			1												Clubbed down
			1												Anophthalmia, unilateral, dysallilognathia
			1												Head - agenesis, liver degeneration
20.0	104	4	1												Anophthalmia - unilateral, dysallilognathia, brain malacia
			1												Head - agenesis
			1												Twins, brain malacia
			1												Anophthalmia - unilateral
10.0	105	5	1												Anophthalmia-unilateral, dysallilognathia
			1												Head agenesis
			2												Anophthalmia - unilateral, encephalocele, dysallilognathia
			1												Head agenesis, spinal cord malacia, liver cytoplasmic degeneration
2.0	103	1	1												Fusion failure - yolk sac
0.2	100	3	2												Anophthalmia, encephalocele, dysallilognathia, celosomia

TERATOGENIC FINDINGS

[illegible]

TABLE 8

AMARANTH
Chi-Square Analyses
H-L-S-V Abnormalities

Dose, mg/kg	Air Cell		Yolk	
	0 hrs	96 hrs	0 hrs	96 hrs
0.004	0.21	-	-	-
0.02	0.06	-	-	-
0.20	0.02	0.01	0.31	0.00
2.00	0.19	0.29	0.00	0.00
10.00	0.60	0.01	1.54	0.51
20.00	0.05	0.32	0.27	0.53
40.00	0.03	0.44	0.24	0.00
All doses (DF)	4.13(7)	1.90(5)	6.30(5)	5.92(5)

AMARANTH IN WATER

% ABNORMAL EMBRYOS

YOLK - 96 HOURSStatistical
Test

Yolk - 96 Hours

Linear Regression Chi-Square

Linearity (DF)	3.989*(1)
Departure (DF)	8.499 (10)
Total (DF)	12.488 (11)
B	0.000603
Standard Error of B	0.0003019

Log Transformation - Chi-Square

Linearity (DF)	5.764*(1)
Departure (DF)	6.723 (10)
Total (DF)	12.488 (11)
B	0.013095
Standard Error of B	0.00545

Dose vs. Ancsin % Abnormal

T	2.86*
B	0.00292
Estimate	0.0508

Log Dose vs. Ancsin % Abnormal

T	3.385*
B	0.0698
Estimate	0.0568

Log Dose vs. % Abnormal

T	2.94*
B	0.0133
Estimate	0.0125

TABLE 6

AMARANTH IN WATER
STATISTICAL ANALYSES
% MORTALITY

Statistical Test	Air Cell		Yolk	
	0 Hours	96 Hours	0 Hours	96 Hours
<u>Linear Regressions</u>				
Log Dose vs. Probit	NS	NS	NS	NS
Dose vs. % Mortality	NS	NS	NS	NS
Dose vs. Arcsin % Mortality	NS	NS	NS	NS
Log dose vs. Arcsin % Mortality	NS	NS	NS	NS
Log dose vs. % Mortality	NS	NS	NS	NS
One-way Analysis of Variance	NS	NS	NS	NS
Linear Regression - Chi Square	NS	NS		
Linearity (DF)			3.978*(1)	16.02*(1)
Departure (DF)			57.104*(10)	37.341*(10)
Total (DF)			61.08*(11)	53.362*(11)
B			0.002755	0.00402
Standard Error of B			0.0013812	0.001005
Log Transformation - Chi Square -				
Linearity (DF)			28.137*(1)	9.936*(1)
Departure (DF)			32.946*(10)	43.426*(10)
Total (DF)			61.08*(11)	53.362*(11)
B			0.1337	0.0572
Standard Error of B			0.0252	0.01815

TABLE 5

AMARANTH IN WATER
STATISTICAL ANALYSES
CHI-SQUARE

Dose mg./kg.	Air Cell		Yolks	
	0 hours	96 hours	0 hours	96 hours
<u>Mortality</u>				
0.004	1.201	-	-	-
0.02	7.215*	-	-	-
0.20	2.978	0.135	0.296	1.699
2.00	1.174	0.225	7.794*	0.896
10.00	0.861	0.456	12.094*	0.068
20.00	5.839*	0.073	9.007*	3.798
40.00	1.469	1.630	5.809*	11.963*
All Doses (DF)	9.821 (7)	5.892 (5)	20.884*(5)	21.589*(5)
<u>% Abnormal</u>				
0.004	0.018	-	-	-
0.02	0.154	-	-	-
0.20	2.059	0.322	0.307	-
2.00	0.124	0.286	0.486	-
10.00	0.603	0.322	1.544	0.505
20.00	3.988*	0.322	0.839	1.395
40.00	0.033	0.003	0.781	0.495
All Doses (DF)	9.882 (7)	0.341 (5)	4.693 (5)	7.759 (5)

* Probablility 0.05 - 0.005.

TABLE 4

AMARANTH in WATER

SUMMARY

YOLK - 96 HOURS

Dose, ppm	No. Fertile	Mortality %	Abnormal			Abnormalities by category						Response % #	Functiona % #
			Total %	#	H-S-V-L %	#	Head % #	Skeletal % #	Viscera % #	Limbs % #	Struc- tural % #		
40.0	102	27.45	1.96	2	0.98	1				0.98	1		0.98
20.0	99	18.18	3.03	3	2.02	2	2.02	2					1.01
10.0	101	7.92	1.98	2	1.98	2	1.98	2					
2.0	100	13.00	0		0								
0.2	102	14.70	0		0								
0.0	101	7.92	0		0								
reced	101	11.88	0		0								
reated	426	10.03	0.23	1	0								0.23

SUMMARY - ALL DOSE LEVELS

504	16.27	82	1.39	7	0.99	5	0.79	4		0.20	1		0.40
-----	-------	----	------	---	------	---	------	---	--	------	---	--	------

TABLE 3

AMARANTH in WATER

SUMMARY

YOLK - 0 HOURS

Dose, ppm	No. Fertile	Mortality % #	Abnormal			Abnormalities by category								Response % #	Functiona % #
			Total % #	H-S-V-L % #	Head % #	Skeletal % #	Viscera % #	Limbs % #	Struc- tural % #						
40.0	107	48.59 52	3.73 4	2.80 3	1.86 2		0.93 1			0.93 1					
20.0	104	52.88 55	3.84 4	2.88 3	2.88 3								0.96		
0.0	105	56.19 59	4.76 5	4.76 5	4.76 5										
2.0	103	51.45 53	0.97 1	0									0.97		
0.2	100	36.00 36	3.00 3	3.00 3	3.00 3										
0.0	105	31.42 33	0.95 1	0.95 1	0.95 1										
rced	104	50.00 52	0	0											
reated	426	11.03 47	0.23 1	0									0.23		

SUMMARY - ALL DOSE LEVELS

519	49.13 255	3.28 17	3.47 18	2.50 13		0.19 1	0.19 1	0.39
-----	-----------	---------	---------	---------	--	--------	--------	------